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Brief communication

Patients with systemic lupus erythematosus and secondary antiphospholipid syndrome have decreased numbers of circulating CD4⁺CD25⁺Foxp3⁺ Treg and CD3⁺CD19⁺ B cells

Ester Rosári Raphaelli Dal Ben^{a,*}, Carine Hartmann do Prado^a,
Talita Siara Almeida Baptista^a, Moisés Evandro Bauer^a, Henrique Luiz Staub^b

^aLaboratory of Immunosenescence, Institute of Biomedical Research, Faculty of Biosciences, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

^bRheumatology Department, Hospital São Lucas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

Introduction: CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cell depletion has been reported in systemic lupus erythematosus (SLE) and, recently, in primary antiphospholipid syndrome (APS); the issue has not been studied in SLE patients with secondary APS (SLE/APS) so far. **Objective:** To quantify total lymphocytes, Treg cells, CD3⁺CD19⁺ T cells and CD3⁺CD19⁺ B cells in SLE/APS patients and healthy controls.

Methods: Cell subtypes underwent immunophenotyping using specific monoclonal antibodies (anti-CD3 CY5, anti-CD4 FITC, anti-CD25, anti-Foxp3, anti-CD19 PE) and flow cytometry. **Results:** Twenty-five patients with SLE/APS (mean age 43.5 years, 96% females, 96% caucasians, mean duration of disease 9.87 years, mean SLEDAI 10 ± 5.77) and 25 age and sex-matched controls entered the study. It was realized that the numbers of Treg and CD3⁺CD19⁺ B cells were significantly lower in SLE/APS patients than in controls (all $p < 0.05$). Treg and CD3⁺CD19⁺ B cells remained numerically low after controlling (ANCOVA) for percentage of total lymphocytes ($p < 0.05$). Decreasing levels of circulating Treg and CD3⁺CD19⁺ B cells correlated to higher scores of lupus activity ($r_s = -0.75$, $p < 0.0001$; $r_s = -0.46$, $p = 0.021$, respectively). Number of Treg cells and CD3⁺CD19⁺ B lymphocytes did not significantly differ in users or nonusers of chloroquine, azathioprine and corticosteroids (all $p > 0.05$).

Conclusions: In this preliminary study, patients with SLE and secondary APS showed depletion of Treg and CD3⁺CD19⁺ B cells; decreasing numbers of both subtypes correlated to a higher SLEDAI. Treg cells depletion might contribute to the autoimmune lesion seen in patients with SLE/APS. The reduced number of CD3⁺CD19⁺ B cells seen in these patients deserves more studies in order to get further elucidation.

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* Corresponding author.

E-mail: esterdalbem@ig.com.br (E.R.R. Dal Ben).

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Pacientes com lúpus eritematoso sistêmico e síndrome antifosfolípide secundária possuem números reduzidos de células B CD4⁺ CD25⁺ Foxp3⁺ (células Treg) e células B CD3⁺ CD19⁺ circulantes

R E S U M O

Palavras-chave:

Lúpus eritematoso sistêmico (LES)
Síndrome antifosfolípide secundária (SAFS)
Células Treg
Células B CD3⁺ CD19⁺

Introdução: A depleção de células T CD4⁺ CD25⁺ Foxp3⁺ regulatórias (células Treg) foi descrita em pacientes com lúpus eritematoso sistêmico (LES) e, recentemente, na síndrome antifosfolípide (SAF) primária; até o momento, o tópico não tinha sido estudado em pacientes com LES e com SAF secundária (LES/SAFS).

Objetivo: Quantificar linfócitos totais, células Treg, células T CD3⁺ CD19⁺ e células B CD3⁺ CD19⁺ em pacientes com LES/SAF e em controles saudáveis.

Métodos: Subtipos celulares foram imunofenotipados utilizando anticorpos monoclonais específicos (anti-CD3CY5, anti-CD4FITC, anti-CD25, anti-Foxp3, anti-CD19PE) e citometria de fluxo.

Resultados: Participaram do estudo 25 pacientes com LES/SAF (média de idade 43,5 anos, 96% mulheres, 96% da raça branca, duração média da doença 9,87 anos, SLEDAI médio 10±5,77) e 25 controles compatibilizados para idade e gênero. Foi constatado que os números de células Treg e de células B CD3⁺ CD19⁺ estavam significativamente mais baixos em pacientes com LES/SAF, em comparação com controles (todos $p < 0,05$). As células Treg e as células B CD3⁺ CD19⁺ permaneceram numericamente baixas em seguida ao controle (ANCOVA) para percentual de linfócitos totais ($p < 0,05$). Níveis decrescentes de células Treg e células B CD3⁺ CD19⁺ circulantes tiveram correlação com escores mais elevados de atividade lúpica ($rs = -0,75$, $p < 0,0001$; $rs = -0,46$, $p = 0,021$, respectivamente). Os números de células Treg e de células B CD3⁺ CD19⁺ não diferiam significativamente em usuários ou não usuários de cloroquina, azatioprina e corticosteroides (todos $p > 0,05$).

Conclusões: Nesse estudo preliminar, pacientes com LES e com SAF secundária demonstraram depleção de células Treg e de células B CD3⁺ CD19⁺; a redução numérica dos dois subtipos teve correlação com aumento de SLEDAI. A depleção de células Treg pode contribuir para a lesão autoimune observada em pacientes com LES/SAFS. O número reduzido de células B CD3⁺ CD19⁺ observado nesses pacientes está a merecer estudos objetivando um aprofundamento em sua elucidação.

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Introduction

Systemic lupus erythematosus (SLE) is a multiorgan disease characterized by immune dysregulation; loss of tolerance to self-antigens leads to formation of immune complexes.¹ Recent data in both human SLE and mouse models have highlighted the role of type I interferon's (α/β) in the initiation and perpetuation of the disease.²

Individuals with SLE are more susceptible to thrombosis than the general population, and the antiphospholipid syndrome (APS) is a well-known risk factor for vascular obstruction in such patients.³ The reason why patients with SLE and APS produce pathogenic antiphospholipid (aPL) antibodies has been a matter of intense debate.

CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells are a distinct thymically derived or inducible subset of T cells with unique immunosuppressive abilities. Quantitative or functional impairment of Treg lymphocytes seems to direct the immune system toward autoimmunity.⁴ It is frequently observed an imbalance between IL-17 producing T helper (Th17) and Treg cells during the course of SLE, with marked impairment of the Treg subset.⁵ It is noteworthy that the blockage of Treg cells by interferon- α -producing antigen presenting cells may contribute to loss of peripheral tolerance in SLE.⁶

A CD4⁺CD25⁺Foxp3⁺ Treg cell dysfunction has been documented in a number of autoimmune disorders.⁷ In SLE cases, most studies have shown decreased number of circulating Tregs in active disease, but data are still conflicting.⁸

B lymphocytes, in turn, are pivotal cells in SLE; they are linked to antigen-presentation, autoantibody synthesis and cytokine production. It is noteworthy that a distinct subset of B cells shown to exert immunosuppressive effects;⁹ these IL-10-producers and regulatory-B cells, knowingly able to suppress T helper cells differentiation, appear to be functionally impaired in SLE patients.¹⁰

Considering the importance of the interplay of T, Treg cells and B lymphocytes in the immunopathogenesis of SLE (5-7; 9,10) we set up to originally quantify total lymphocytes, Treg cells, CD3⁺CD19⁺ T cells and CD3⁺CD19⁺ B cells in a Brazilian survey of patients with SLE/ secondary APS (SLE/APS) and healthy controls.

Material and methods

This cross-sectional study included patients with SLE and history of secondary APS from our Outpatient Lupus Clinic. Clinical and laboratory diagnosis of SLE were based on the American College of Rheumatology 1997 criteria,¹¹ while the

Sidney 2006 criteria was utilized to diagnose APS.¹² Lupus activity was assessed by the systemic lupus erythematosus disease activity index, SLEDAI.¹³

The exclusion criteria were as follows: 1) age below 16 years; 2) infective endocarditis or other current infections; 3) diabetes mellitus; 4) neoplasms (current or past); and 5) infection by human immunodeficiency virus or *Treponema pallidum*. The control group comprised healthy individuals aged more than 16 years-old, matched by age and sex, and with no APS, connective tissue disorders, neoplasms or current infections. Clinical and demographic data were obtained from a chart review and interview with patients or family after informed consent. The study was approved by the local ethics committee.

Cell subtypes underwent immunophenotyping using the specific monoclonal antibodies anti-CD3CY5, anti-CD4 FITC, anti-CD25, anti-Foxp3 and anti-CD19 PE (Biosciences, San Jose, CA, USA) and identified by multicolor flow cytometry.¹⁴

Quantitative statistical analysis was performed using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). The significance level was set at $\alpha = 0.05$ (two-tailed). The results were shown as means and standard deviation or by absolute and relative frequencies. The statistical analysis was performed by Student's t test and the Mann-Whitney test for the continuous variables, and chi-square or Fisher's exact test for categorical variables. The Pearson test was utilized to correlate circulating cell subtypes with the SLEDAI. An analysis of covariance (ANCOVA) was performed to evaluate relationships between variables.

Results

Twenty-five patients with SLE/APS and 25 healthy controls entered the study. Middle-aged (mean age 43.5 years) females (96%) highly predominated in our SLE/APS survey. Twenty-four patients (96%) were caucasians. Mean duration of the disease was 9.87 years, and the average SLEDAI score was 10 ± 5.77 . At the moment of evaluation, arthritis (36%), oral ulcers (32%), malar rash (24%), seizures (16%), nephritis (12%), and leukopenia (8%) were the most frequent SLE manifestations. All patients had antinuclear antibodies,

and 40% were positive for anti-dsDNA. Low complement levels were seen in 8% of patients.

Regarding the APS clinical features, deep vein thrombosis (DVT) was seen in 15 patients (60%), while fetal losses (so considered after 12 weeks of pregnancy) occurred in 6 patients (24%). Optic neuritis was seen in 4 cases (16%). Stroke and miscarriages were each diagnosed in 3 cases (12%). Moderate or high levels of anticardiolipin (aCL) antibodies were detected in 21 patients (84%), whereas lupus anticoagulant was present in 9 patients (36%). Fourteen patients (56%) were on oral anticoagulation regime with warfarin, and the remaining was being treated with low-dose aspirin.

Chloroquine, azathioprine and corticosteroids were being utilized by 9 patients (36%), 5 patients (20%) and 16 patients (64%), respectively.

Table 1 compares demographic aspects and lymphocyte subsets of SLE/APS patients and controls. Both groups were homogenous regarding age, gender and race. The mean number of CD4⁺CD25⁺Foxp3⁺ Treg cells and CD3⁺CD19⁺ B cells were significantly lower in patients with SLE/APS when compared to controls. The mean number of total lymphocytes, CD3⁺CD19⁺ T cells and CD4⁺CD25⁺ lymphocytes did not significantly differ between groups.

The SLE/APS patients were maintaining a significantly lower number of Treg cells after the control (ANCOVA) for percentage of total lymphocytes ($F = 28.50$, $p < 0.0001$). The FoxP3 expression in CD4⁺CD25⁺ cells, as estimated by mean fluorescence intensity, did not differ in SLE/APS patients and controls (2660.55 ± 1044.06 vs 2470.65 ± 1732.87 ; $p = 0.67$; respectively). Patients with SLE/APS persisted with significantly lower percentage of CD3⁺CD19⁺ B cells after controlling for total lymphocytes ($F = 13.17$; $p = 0.002$).

Fig. 1 shows the distribution of total lymphocytes, CD4⁺CD25⁺Foxp3⁺ Treg cells and CD3⁺CD19⁺ B lymphocytes in SLE/APS patients as compared to controls.

The Pearson test for correlation of circulating Treg and CD3⁺CD19⁺ B lymphocytes with the SLEDAI is shown in Fig. 2. A significant negative correlation of both cell subtypes with the SLEDAI was obtained, indicating that a lower number of Treg and CD3⁺CD19⁺ B cells were linked to increasing scores of lupus activity.

Table 1 – Demographic data and lymphocyte subset in 25 patients with systemic lupus erythematosus/secondary antiphospholipid syndrome (SLE/APS) and 25 healthy controls

	SLE/APS		Controls		p
		(n = 25)		(n = 25)	
Mean age (years \pm SD)		43.5 \pm 12.84		43.80 \pm 8.45	0.93 ^a
Females		24 (96%)		24 (96%)	1.00 ^a
Caucasians		24 (96%)		24 (96%)	1.00 ^a
Total lymphocytes	29.42 \pm 4.57%	2227.86 \pm 528.83 cells/ μ L	32.2 \pm 2.49%	2523.60 \pm 528.83 cells/ μ L	0.13 ^b
CD3 ⁺ CD19 ⁺ T lymphocytes	73.72 \pm 8.34%	1522.52 \pm 527.20 cells/ μ L	73.64 \pm 7.70%	858.38 \pm 194.32 cells/ μ L	0.100 ^b
CD4 ⁺ CD25 ⁺ T lymphocytes	1.28 \pm 0.89%	20.50 \pm 9.35 cells/ μ L	1.81 \pm 0.80%	45.68 \pm 20.19 cells/ μ L	0.81 ^b
CD4 ⁺ CD25 ⁺ Foxp3 ⁺ T lymphocytes	0.74 \pm 0.34%	21.61 \pm 12.70 cells/ μ L	1.83 \pm 0.77%	46.18 \pm 19.43 cells/ μ L	<0.0001 ^b
CD3 ⁺ CD19 ⁺ B lymphocytes	5.71 \pm 2.66 %	136.34 \pm 92.68 cells/ μ L	9.25 \pm 3.00%	233.43 \pm 75.71 cells/ μ L	0.006 ^b

SD, standard deviation.

^aChi-square test.

^bStudents t test.

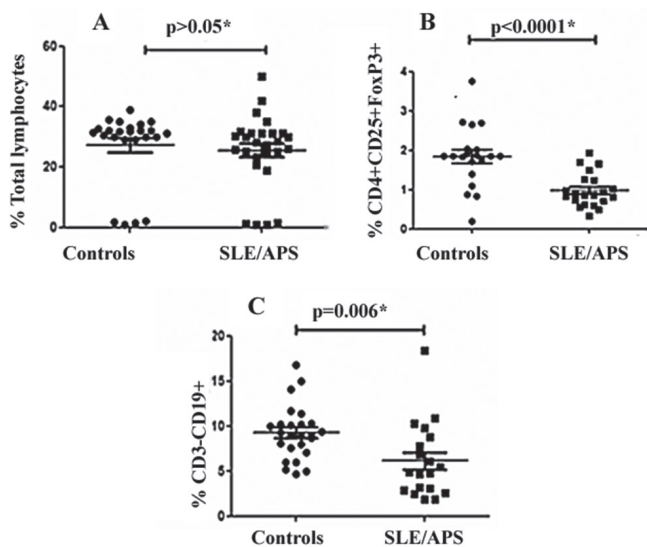


Fig. 1 – Graphical distribution of total lymphocytes (A), CD4+CD25+Foxp3+ Treg cells (B) and CD3-CD19+ B cells (C) in controls and patients with systemic lupus erythematosus/secondary antiphospholipid syndrome (SLE/APS). *Student's t-test.

Levels of circulating Treg cells did not significantly vary among users or nonusers of chloroquine, azathioprine and corticosteroids ($p = 0.90$; $p = 0.76$ and $p = 0.29$ in the chi-square test, respectively). Similarly, the number of CD3-CD19+ B cells did not significantly differ in users on nonusers of these medicines ($p = 0.462$; $p = 0.512$ and $p = 0.341$ in the chi-square test, respectively).

When we compared cell subtypes selectively in 5 patients with inactive SLE/APS (SLEDAI < 4) and 25 healthy controls, only the CD3-CD19+ B population remained diminished in the first group ($5.37 \pm 1.95\% \times 9.25 \pm 3.00\%$; $p = 0.003$; Student's t test).

Discussion

A number of reports have accounted for a decrease of Treg cells in patients with SLE.¹⁵⁻¹⁷ As far as we are aware, this study is the first to approach circulating Treg cells and B lymphocytes in Brazilian patients with SLE and secondary APS. Our survey included predominantly middle-aged white females with high SLEDAI, and mean disease duration of approximately a decade. Arthritis and oral ulcers were the prominent SLE findings, while DVT and aCL antibodies were cardinal APS features.

We have found a decreased number of circulating CD3-CD19+ B cells in our SLE/APS patients when compared to controls. It is important to say that there was no association of CD3-CD19+ B cell count with intake of chloroquine, azathioprine and steroids. The reasons for this B cell decrease in SLE/APS patients are nebulous, but an explanation could be auto-antibodies directed to B lymphocytes or intrinsic defects of B cell subsets. The fact that B cell depletion was confirmed even in the 5 patients with inactive SLE might support the latter.

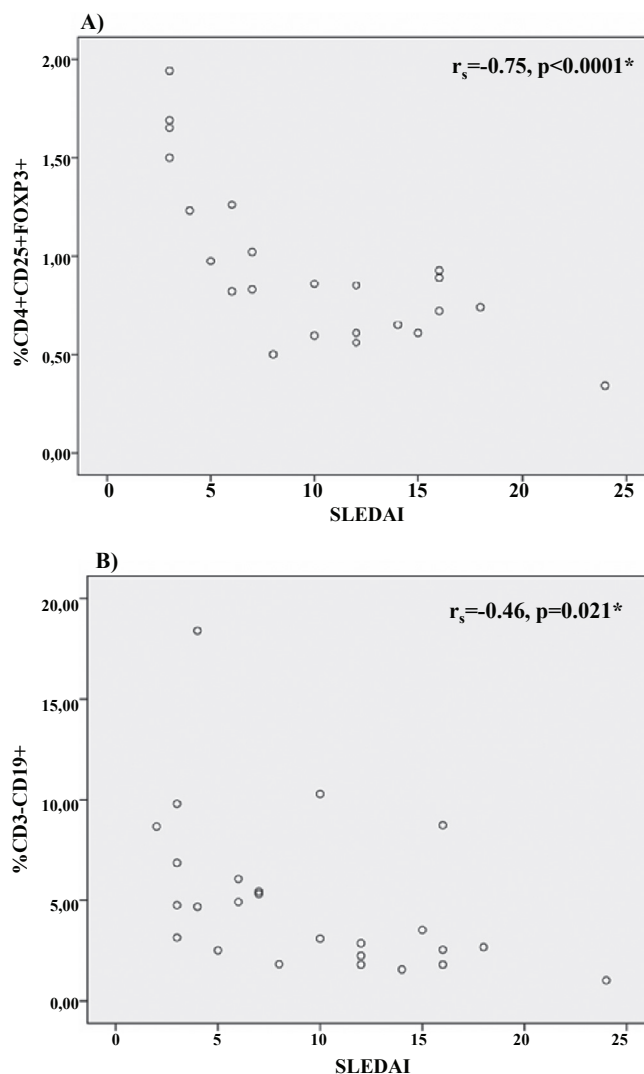


Fig. 2 – Pearson correlation of circulating Treg cells (A) and CD3-CD19+ B cells (B) with lupus activity assessed by the systemic lupus erythematosus disease activity index (SLEDAI). *Student's t test

We could only suppose the hypothesis that the population with B cells depletion is the IL-10 producer subset with immunosuppressive properties. It is noteworthy that competent B cells seem to be important to trigger Treg activity, as shown in patients with common variable immunodeficiency¹⁸.

Reduced numbers of circulating CD4+CD25+Foxp3+ Treg cells were seen in our SLE/APS patients as compared to controls. As occurred the with the B cell subtype, Treg cells depletion was confirmed after controlling (ANCOVA) for total lymphocyte count. Decreasing levels of circulating Treg cells, just as seen with the CD3-CD19+ B subset, were correlated with higher scores of disease activity. Diminished levels of Treg cells have been associated with active SLE^{19,20}, but other reports^{21,22} questioned this finding. In our study, Treg cells depletion could be representative of an intrinsic defect related to previous SLE/APS, or current SLE activity. Patients with inactive SLE did not show Treg reduction, which favors the second hypothesis. It is worthy of note that there was no association between Treg

cells decrease and the intake of chloroquine, azathioprine or steroids in our patients either.

Our group recently reported low levels of circulating Treg and also of CD3-CD19⁺ B cells in patients with primary APS²³. These data, combined with the current study, imply that depletion of both subsets occurs in APS populations as a whole. If this assumption will be confirmed, the Treg cells decrease may comprise one of the immune mechanisms leading to pathogenic aPL responses in primary or secondary APS. Nevertheless, it should be considered that Treg cells decrease in our SLE/APS patients seemed to relate more to SLE activity than any other particular factor.

Recent data disclosed that any quantitative analysis of Tregs in patients with autoimmune disorders have to be seen with some caution. The reg cells show formidable plasticity and comprise a heterogeneous population of suppressive cells, non-functional Treg cells and IL-17A-producing Treg cells acting as effector T lymphocytes. Thus, the simple numeric count of Tregs might not be truly representative of their functional status.²⁴

Some other limitations of our study must be mentioned. Even though the majority of our patients showed active SLE, the APS thrombotic manifestations were not current; newer studies should investigate the biological function of Treg cells and B lymphocytes during the thrombotic event and also in a longitudinal analysis. Given the small sample, we could not subgroup patients by SLE/APS features or drug dosage. The small number of patients with inactive SLE restricted the statistical power of the article. The same way, the lack of comparison between patients with SLE and without APS limited our conclusions.

Up to date, the treatments of SLE and APS have been reasoned in immunosuppression and anticoagulation, respectively. A direct immunomodulatory approach shifting the balance to favor Treg cells is being tried with autologous Treg cell therapy in type-1 diabetes,²⁵ and such intervention might be promising for SLE and APS as well. Recently, it was noticed that Treg cells were able to prolong the interval of remission induced by conventional cytostatic drugs in (NZB × NZW) F1 lupus mice.²⁶

Although many questions concerning the pathogenesis of SLE and APS remain undefined, it is possible that the progression of the disease results from a breakdown in Treg-dependent peripheral self-tolerance. In this context, Treg-based immunotherapy might have a place in the maintenance of disease remission in this group of patients.

In summary, this preliminary study demonstrated impaired numbers of CD4⁺CD25⁺Foxp3⁺ Treg cells and CD3-CD19⁺ B lymphocytes in Brazilian patients with SLE and secondary APS. Lower cell counts were seen in patients with higher SLE activity. Future studies shall confirm if the decrease of Treg cell levels is connected to the abnormal immune response seen in SLE/APS. The decrease of CD3-CD19⁺ B cells seen in these patients, and potentially linked to Treg dysfunction, also justifies new studies in order to search for more clarifications.

Conflicts of interest

The authors declare no conflicts of interest.

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